REMARKS/ARGUMENTS

The Pending Claims

Claims 22, 23, 25, 26, and 28-64 are pending and are directed to a liposome encapsulating a water-soluble substance in an internal cavity thereof, wherein the liposome has a particle size of 300 nm or less (claims 22, 23, 28-45, and 47-63), a composition comprising the liposome (claims 46 and 64), and a method of producing the liposome (claims 25 and 26).

Claims 25 and 26 have been labeled as withdrawn because the Examiner considers that the claims are directed to a non-elected invention in response to a restriction requirement. Since claims 25 and 26 are directed to a method of producing the elected product, Applicants request that any withdrawn claims be rejoined in accordance with the provisions of MPEP § 821.04 upon the allowance of one or more of the elected product claims to the extent such withdrawn claims depend from or otherwise include all of the limitations of an allowed claim. In the event of rejoinder, the requirement for restriction between the product claims and the rejoined method claims should be withdrawn, and the rejoined method claims should be fully examined for patentability in accordance with 37 CFR § 1.104.

Amendments to the Claims

Claims 1-21, 24, and 27 have been canceled. Claims 22 and 25 have been amended to remove the dependency on claim 1 (which claim has been canceled) and to incorporate the features of claim 24 and 27 (now canceled), respectively. Claim 28 is new and is similar to claim 22, except that the method of producing the liposome is not recited therein.

Claims 29-36, 38-54, and 56-64 are new and recite similar subject matter as claims 2 and 5-21 (now canceled). Claims 37 and 55 are new and are supported by the specification at, for example, page 9, lines 6-7.

No new matter has been added by way of these amendments to the claims.

Summary of the Office Action

The Office maintains the rejection of claims 1-5, 7, 11-16, 20, and 21 and newly rejects claims 22-24 under 35 U.S.C. § 102(b) as allegedly anticipated by Modi (U.S. Patent 6,193,997).

The Office maintains the rejection of claims 1-7 and 9-24 under 35 U.S.C. § 103(a) as allegedly obvious in view of Modi and Slater et al. (U.S. Patent Application Publication 2003/0133973). Additionally, the Office maintains the rejection of claims 1-5, 7, 8, and 11-24 under 35 U.S.C. § 103(a) as allegedly obvious in view of Modi and Tagawa et al. (EP 1170018).

In the Advisory Action dated October 20, 2009, the Office contends that the recitation that the liposome has a particle size of more than 10 nm and less than 300 nm is not supported by the specification and, therefore, is considered to be new matter.

Reconsideration of these rejections is hereby requested.

Discussion of the Anticipation Rejection

The Office contends that Modi discloses a mixed liposome comprising a triglycerol and, therefore, anticipates the subject matter of claims 1-5, 7, 11-16, and 20-24. This rejection is traversed for the following reasons.

The pending claims, as amended, are directed to a liposome encapsulating a water-soluble substance in an internal cavity thereof, wherein the liposome has a particle size of 300 nm or less, as well as a composition comprising the liposome and methods for preparing the liposome. Claims 28 and 47-64 recite that the liposome comprises a triglycerol and cholesterol, wherein claims 22, 23, 25, 26, and 29-46 recite that the liposome is produced by a particular process. The process includes dissolving a phospholipid, a triglycerol, and cholesterol in a water-immiscible organic solvent, mixing the resulting solution with an aqueous solution of the water-soluble substance, emulsifying the mixture to prepare a W/O emulsion with a particle size of 10 to 150 nm, adding the W/O emulsion in an aqueous phase with stirring to form a double emulsion (W/O/W emulsion), and removing the organic solvent from the double emulsion to form a liposome. The liposome prepared by this process

contains triglycerol and cholesterol and has a particle size that is greater than the W/O emulsion particle size (i.e., greater than 10 nm).

The structure resulting from the process steps is to be considered when assessing the patentability of the product defined by product-by-process claims over products described in the prior art, as is the situation with claims 22, 23, and 29-46. See, e.g., M.P.E.P. § 2113 ("The structure implied by the process steps should be considered when assessing the patentability of product-by-process claims over the prior art, especially where the product can only be defined by the process steps by which the product is made, or where the manufacturing process steps would be expected to impart distinctive structural characteristics to the final product.").

The inventive liposome differs from the mixed liposome of the Modi reference because the inventive liposome contains cholesterol, which is not disclosed by the Modi reference. Therefore, the Modi reference cannot be considered to anticipate the subject matter of the pending claims.

Furthermore, the inventive liposome recited in claims 22, 23, 25, 26, and 29-46 has a particle size that is greater than 10 nm, which differs from the mixed liposome of the Modi reference that has a particle size of 10 nm or less. As discussed above, claims 22, 23, 25, 26, and 29-46 define the liposome by a particular process used to produce the liposome, wherein the particle size of the W/O emulsion is 10 to 150 nm. The particle size of a liposome is greater than the corresponding particle size of the W/O emulsion used to make the liposome (e.g., the particle size of the inventive liposome must be *greater* than 10 nm if the smallest possible particle size of the W/O emulsion is 10 nm), as is exemplified by Figure 1 of Ishii, "Production and Size Control of Large Unilamellar Liposomes by Emulsification" at pages 111-121 (Chapter 7) in *Liposome Technology. 2nd Edition. Volume 1: Liposome Preparation Techniques and Related Techniques*, Gregory Gregoriadis (editor), CRC Press, Inc., 1993 (submitted herewith). In Figure 1, a W/O emulsion is added to water to prepare a W/O/W emulsion. An organic solvent in the W/O/W emulsion is evaporated using an aspirator to obtain a liposome. In the procedure depicted in Figure 1, the aqueous phase in the W/O emulsion is present in oil particles of the W/O/W emulsion, which are encapsulated

within the liposome. Accordingly, the particle size of a resulting liposome is larger than the particle size of the W/O emulsion used to produce the liposome.

This point is further supported by the Declaration Under 37 C.F.R. § 1.132 of Toshiaki Tagawa, Ph.D. dated January 18, 2010, which is submitted herewith. In the Rule 132 Declaration, Dr. Tagawa identifies the particle sizes of the W/O emulsions corresponding to the liposome particle sizes disclosed in Example 7 and Fig. 2B of the specification of the present application. As shown in the table on page 3 of the Rule 132 Declaration, the particle size of the liposome was greater than the particle size of the W/O emulsion in every case.

Thus, for the additional reason that the liposome recited in claims 22, 23, 25, 26, and 29-46 has a particle size that is greater than 10 nm (i.e., greater than the particle size of the mixed liposome of the Modi reference), the subject matter of claims 22, 23, 25, 26, and 29-46 is not anticipated by the Modi reference.

Accordingly, Applicants request that the anticipation rejection be withdrawn.

Discussion of the Obviousness Rejections

The Office maintains its contention that it would have been obvious for one of ordinary skill in the art to arrive at the claimed invention in view of the combined disclosures of the Modi reference and either of the Slater or Tagawa references. The obviousness rejections are traversed for the following reasons.

As discussed above, the inventive liposome, composition, and method of producing the liposome differ from the disclosure of the Modi reference by the inclusion of cholesterol in the inventive liposome and – for claims 22, 23, 25, 26, and 29-46 – by a particle size of the inventive liposome of greater than 10 nm.

As discussed in the "Reply to Office Action" dated October 13, 2009, the mixed liposome of the Modi reference differs from the inventive liposome and the liposomes disclosed in the Slater and Tagawa references based on particle size. The Slater reference discloses liposomes with particles sizes of 40-250 nm (see, e.g., paragraph 82 of the Slater reference), and the Tagawa reference discloses liposomes with particles sizes of 20-500 nm (see, e.g., paragraphs 22-23 of the Tagawa reference). Accordingly, the Modi reference and

the Slater and Tagawa references are directed to two different products ("mixed liposomes" versus conventional liposomes), such that one of ordinary skill in the art would not have had any reason to combine the disclosures of these references, let alone in the particular manner that would result in the claimed invention.

Moreover, even if one of ordinary skill in the art did combine the disclosures of the cited references, one of ordinary skill in the art would not necessarily have arrived at the claimed invention. In particular, the Modi and Slater references do not teach or suggest using cholesterol in the liposome, as required by the pending claims.

The combination of triglycerol and cholesterol provides for an improved thermodynamic stability of the membrane of the liposome, thereby providing for a high rate of encapsulation. In particular, the inventors unexpectedly discovered that the combination of triglycerol and cholesterol provides for a reduced viscocity and uniform dispersion during the process for preparing the liposome. The advantageous effects of the inventive liposome are demonstrated in Example 7 and Figs. 2A and 2B of the present application. In particular, the encapsulation ratio was 70% or higher when triolein was added in the liposome production process (also containing cholesterol) as compared to an encapsulation ratio of 55% obtained in the absence of triolein (see Fig. 2A). Additionally, the addition of triolein together with cholesterol resulted in uniform average particle size of 170 nm, while the average particle size in the absence of triolein was 240 nm.

The existence of the unexpected benefits attendant the present invention further supports the unobviousness of the present invention and rebuts any *prima facie* obviousness position recited in the Office Action assuming arguendo that the combination of the disclosures of the cited references are considered to properly establish *prima facie* obviousness.

For the above-described reasons, the obviousness rejections should be withdrawn.

Discussion of the Potential New Matter Issue Raised in the Advisory Action

Applicants note that claim 1, which recited that the liposome has a particle size of more than 10 nm, as well as claims 2-21 dependent thereon, have been canceled. The remaining claims do not recite that the liposome has a particle size of more than 10 nm

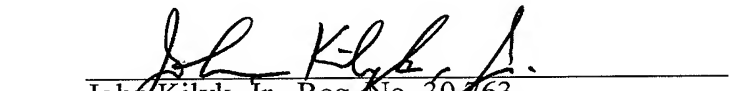
(though this feature is apparent from the preparation method recited in claims 22, 23, 25, 26, and 29-46 as discussed above).

In view of the cancelation of claims 1-21, Applicants believe that the new matter issue no longer is relevant.

Conclusion

Applicants respectfully submit that the patent application is in condition for allowance. If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to call the undersigned attorney.

Respectfully submitted,



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Chapter 7

PRODUCTION AND SIZE CONTROL OF LARGE UNILAMELLAR LIPOSOMES BY EMULSIFICATION

Fumiyoshi Ishii

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I. INTRODUCTION

For more than a decade there has been interest in the production of liposomes as models for biological membranes and as experimental drug carriers. A number of different methods have been developed for the preparation of liposomes. These methods have been classified for convenience into three categories: (1) mechanical dispersion methods such as hand shaking or vortexing,[1] sonication,[2] and use of a French press;[3] (2) detergent-solubilizing dispersion methods including solubilized lecithin dispersion with sodium cholate[4] or octylglucoside;[5] and (3) solvent dispersion methods such as ethanol injection,[6] ether infusion,[7] and reverse-phase evaporation (REV).[8] Liposomes prepared by solvent dispersion methods other than ethanol injection have a higher encapsulation efficiency of the aqueous phase than those prepared by methods in the other two categories. However, with solvent dispersion methods, all liposomes have at least one of several disadvantages: low encapsulation efficiency, manyfold dilution, heterogeneous size and size distribution, restrictions on lipid composition, and limitations in the solubility of lipid in specific organic solvents.

In general, methanol, ethanol, ether (diethyl, isopropyl, petroleum), hexane, benzene, chloroform, dichloromethane, and other hydrocarbons can be used for the preparation of liposomes by solvent dispersion methods. In particular, the last five organic solvents, which are water immiscible, are useful for liposome preparation using a water-in-oil (W/O) emulsion, such as the REV method of Szoka and Papahadjopoulos,[8] the electroemulsification method of Ishii and Noro,[9] and the microencapsulation method of Ishii et al.[10] In these methods, the size of the emulsion droplets, which can be readily controlled by the intensity of mechanical shear or energy, has a direct effect upon the size and size distribution of the resulting liposomes.

The mean size and size distribution of liposomes are important factors affecting physicochemical stability,[11] encapsulation efficiency,[12] tissue distribution,[13] *in vivo* circulation lifetimes,[14] and transfer of lipid onto cells.[15] The sizing of liposomes prepared by various methods can be controlled by membrane filters[16] and a sizing apparatus such as Extruder®[17] and Lipoprep®.[18] On the other hand, several preparations of vesicles with a well-defined size have been produced: sonicated vesicles 250 Å in diameter characterized by Huang,[19] detergent removal vesicles with an average diameter of 300 Å by Brunner et al.,[4] and French press extrusion vesicles 315 to 525 Å in diameter by Barenholz et al.[20] However, the control of particle size distribution within a liposome preparation has not been examined in detail.

In a more recent study,[10] microencapsulation vesicles (MCV) prepared using the emulsification technique were found to be excellent as drug-delivery carriers, showing high encapsulation, stability, and reproducibility. Previous studies using this method have shown that particle size distribution and mean

diameter could be controlled within one order of magnitude by mechanical agitation. The author has now extended this work to study in detail the control of particle size using various lipid solvents with different boiling points.

II. EXPERIMENTAL

A. MATERIALS

Egg phosphatidylcholine (PC) was kindly provided by Asahi Chemical Industries Co., Ltd., Japan. Calcein (Wako Pure Chemical Industries, Ltd.) was chosen as the encapsulated model marker and was purified according to the method of Allen.[21] Diethyl ether (b.p. 34.6°C), dichloromethane (b.p. 39.8°C), chloroform (b.p 61.2°C), *n*-hexane (b.p. 68.7°C), and benzene (b.p. 80.1°C) were used as lipid solvents. These five different lipid solvents and other agents were all extra-pure-grade materials. Distilled water used was deionized.

B. METHODS

1. Preparation of Liposomes

Liposomes were prepared using the microencapsulation method described in a previous paper.[10] Briefly, egg PC (135 m*M*) was dissolved in 10 ml of each of the above five lipid solvents. Then, 5 ml of aqueous phase including 10^{-3} mol/l calcein was added to each lipid solution and emulsified with a sealed-type homogenizer (Type DX-T, Nihonseiki Co., Ltd., Tokyo, Japan) at 7000 rpm for 10 min to form a (W/O) emulsion. This emulsion (15 ml) was then added immediately to ten volumes (150 ml) of water in a spherical reaction flask under agitation with a Chemistirrer (Type B-100, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) at 520 rpm to form a complex water-in-oil-in-water (W/O/W) emulsion. For evaporation of the organic solvent, stirring was then continued at constant temperature (30°C) for 1 h under a stream of nitrogen gas at a flow rate of 10 l/min. The solvent-dissolved lipids were gradually transferred by dissolution into the aqueous phase. Following solvent evaporation from the surface of the aqueous mother liquid, a lipid film was formed. Lipid vesicles (liposomes) were formed at this point. Complete evaporation of the solvent from the system was confirmed by gas chromatography. Free calcein was removed by gel filtration through a Sephadex® G-50 column (2.5 × 40 cm) at room temperature. The procedure for liposome preparation by the MCV method is shown schematically in Figure 1.

2. Electron Microscopy

a. Freeze-Fracture Electron Micrographs

Freeze-fracture electron micrographs were prepared using a modification of the procedure of Bosworth et al.[12] Liposome preparations were mixed with 25% (v/v) glycerol solution and then quickly frozen in Freon® slush at −170°C.

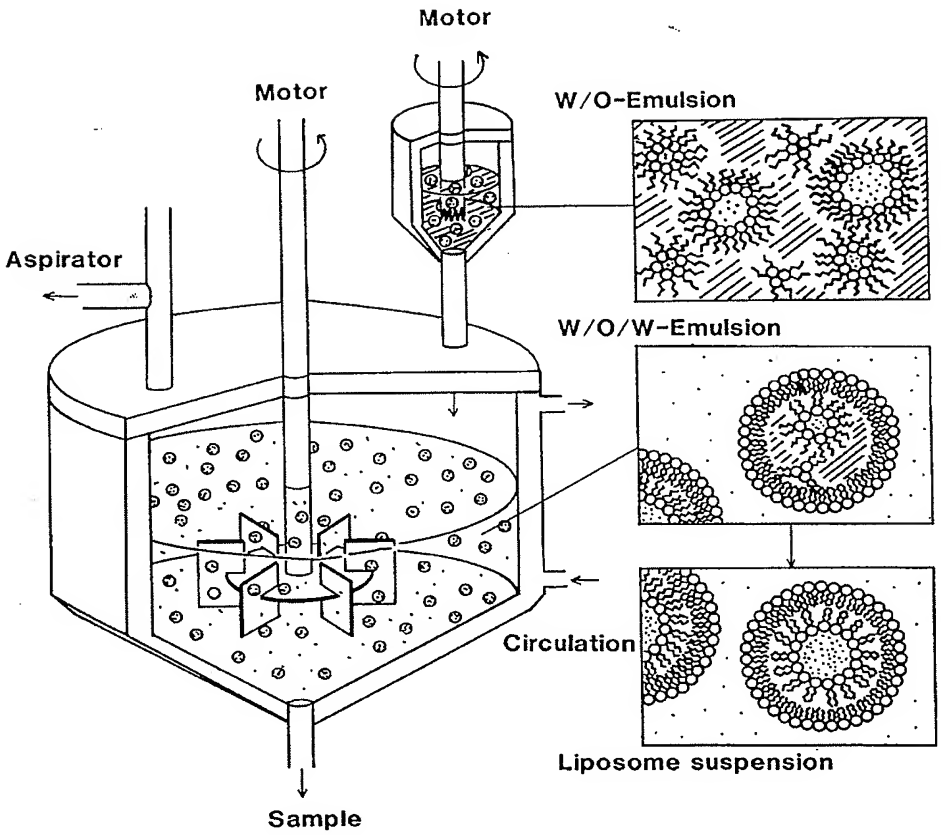

FIGURE 1. Diagrammatic representation of the emulsification process for the preparation of large unilamellar liposomes.

The samples were fractured in a freeze-etching apparatus (Model JFD-9000, JEOL, Japan) at −120°C and replicated by platinum-carbon shadowing. Replicated samples were transferred to flamed 200-mesh grids and observed using a transmission electron microscope (Model JEM-1200EX II, JEOL, Japan).

b. Scanning Electron Micrographs

Scanning electron micrographs were obtained by a specific fixation technique using malachite green described in a previous paper.[22] Briefly, pieces of qualitative filter paper (Whatman® 2) were immersed in the liposomal suspension and then rapidly removed. The liposomes adsorbed on the filter paper were fixed by immersion for 24 h at 4°C in 1% (v/v) glutaraldehyde and 1% (w/v) malachite green mixed in buffer solution ($NaH_2PO_4Na_2HPO_4$, pH 7.4). After fixation, the liposomes were briefly washed in buffer solution

and reacted for 8 h with cold 1% (w/v) osmium tetroxide buffered with phosphate. All samples were subsequently dehydrated in a graded ethanol series. After critical-point drying with liquid carbon dioxide, the fixed liposomes were mounted on a sample stage with double-sided adhesive tape, vacuum-coated with a layer of gold about 300 Å thick, and viewed in a scanning electron microscope (Model JSM-T200, JEOL, Japan).

3. Determination of Liposome Particle Sizes

The particle sizes of liposomes were determined by measuring their diameters on scanning electron micrographs. For each preparation, at least 300 liposome particles were measured in three separate experiments.

4. Measurement of W/O Emulsion Viscosity

W/O emulsion viscosities were measured with a rotating viscometer (Vismetron VSA-1, Shibaura System Co., Ltd., Japan) at 20 ± 0.1°C, employing a circulating water bath.

III. RESULTS AND DISCUSSION

The MCV method of in-liquid drying, a microencapsulation technique capable of encapsulating aqueous solutions of materials such as enzymes, hormones, genetic material, and biologically active peptides, is simple, rapid, versatile, and reproducible. Moreover, water-insoluble material can also be encapsulated in a similar manner.

As shown in Figure 1, the MCV method is essentially different from the REV vesicle method of Szoka and Papahadjopoulos[8] and the lecithin-span 80 mixed vesicle method of Matsumoto et al.,[23] in the step involving evaporation of the solvent from the oil phase-dissolved lipids. In the latter methods, the lipid solvent is removed from the surface of the oil phase of the W/O emulsion, resulting in poor reproducibility of liposome formation. However, in the MCV method, it is possible to obtain fairly good reproducibility with regard to size distribution, encapsulation efficiency, and physical stability in comparison with the other methods, since removal of the solvent is performed by mechanical agitation during preparation of the W/O/W emulsion. Moreover, an important characteristic of the MCV method is that liposomes can be prepared by emulsification without either an emulsifier or a dispersing agent. That is, phospholipid (egg PC) can be used as an emulsifier to prepare the W/O emulsion. Furthermore, the spontaneous swelling of phospholipids that occurs in aqueous solution during preparation of the W/O/W emulsion is exploited.

Freeze-fracture electron micrographs of MCV prepared with various organic solvents are shown in Figure 2. All the micrographs show that each preparation contains spherical particles ranging in size from 50 to 500 nm. Thus, judging from the freeze-fracture photographs, all preparations were

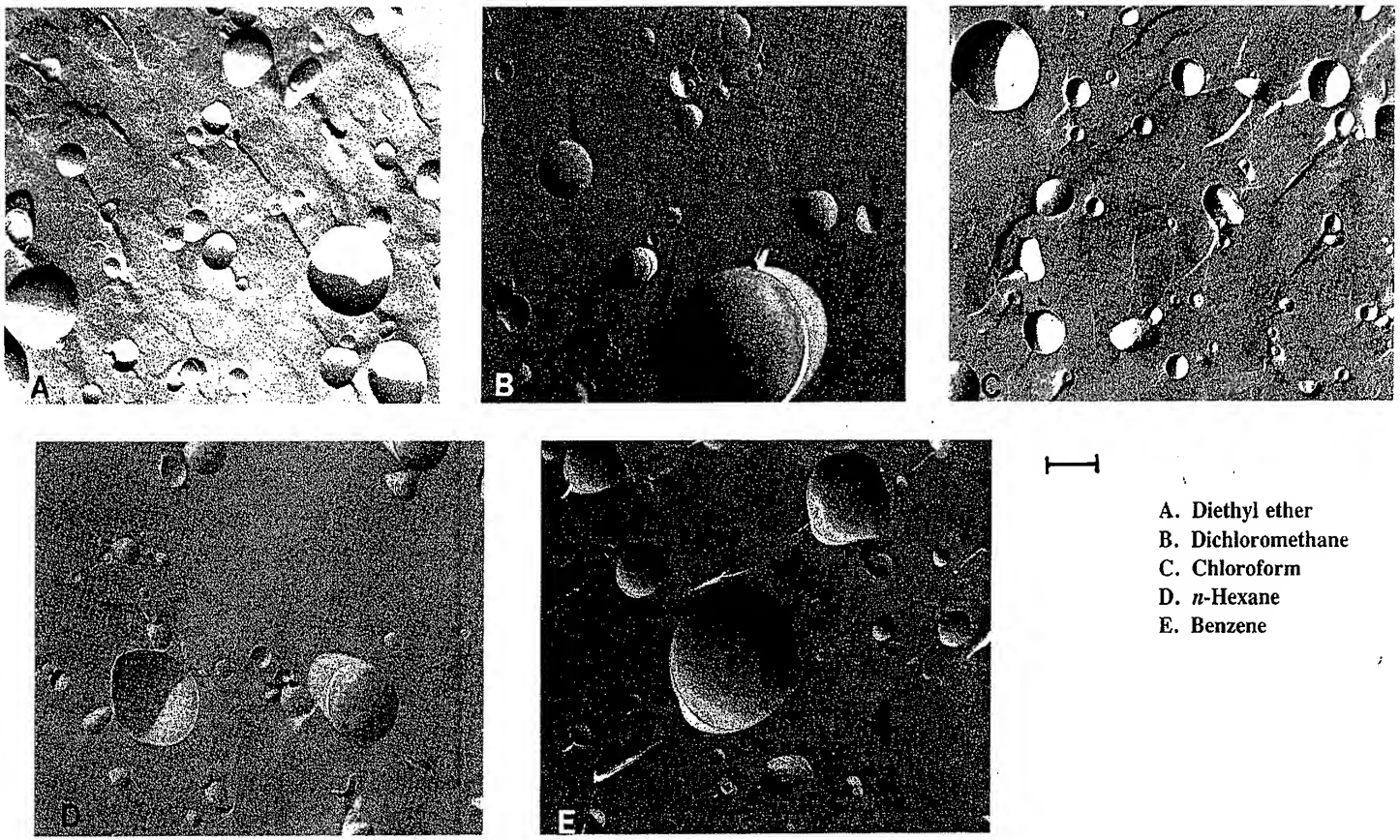

FIGURE 2. Freeze-fracture electron micrographs of MCV prepared with five different organic solvents. Bar indicates 200 nm.

heterogeneous. The principal disadvantage of freeze fracture is that the plane of fracture is not a midplane one, since the liposome particles are randomly positioned in the frozen section. Thus, this technique appears to be unsuitable for the estimation of the mean size and size distribution of liposomes. In all photographs, the structure of the liposome shell was not apparent, suggesting the presence of a single bilayer and, thus, unilamellar vesicles. Consequently, the freeze-fracture technique allows visualization of the internal lamellae of liposomes.

The surface fixation of liposomes with malachite green to observe their steric forms using scanning electron microscopy was originally introduced by Ishii et al.[22] Scanning electron micrographs of various liposomes prepared with different lipid solvents are shown in Figure 3. All the liposome particles were spherical with smooth surfaces. Moreover, it is clear that all preparations showed homogeneity of both size and shape. The sizes of liposomes prepared using lipid solvents with higher boiling points, *n*-hexane (Figure 3d) and benzene (Figure 3e), were larger than those obtained with other solvents. It was reported previously[10] that this specific fixation technique was useful for the observation of the morphology of the liposome surface, especially as it provided a direct approach for the determination of particle size.

Figure 4 shows the relation between the mean diameter of liposomes determined for 900 particles on scanning electron micrographs and the boiling point of lipid solvents used for liposome preparation. As it can be seen, the mean diameter of liposomes decreased linearly as the boiling point of the solvent increased. In the MCV method, the W/O emulsion is poured into a dispersion medium (water), where it spontaneously forms a W/O/W emulsion, then the solvent of the oil phase is gradually lost by dissolution into the aqueous phase, finally producing liposomes. Thus, when removal of the solvent by evaporation occurs too quickly, the liposomes are produced without sufficient dispersion. Hence, the faster the removal of the solvent by evaporation, the larger the size of the resulting liposomes.

The viscosity of the W/O emulsion prepared in this study was also an important factor affecting the size of liposomes. Figure 5 shows this effect. The mean liposome diameter increased linearly as the viscosity of the W/O emulsion increased. In general, it is well known that the effect of emulsion droplet size on the viscosity of an emulsion system increases with decreased mean droplet size. However, it can be easily assumed that an increase in the viscosity of a W/O emulsion would make it difficult for a W/O/W emulsion to form. In this method, as a W/O/W emulsion is formed from a W/O emulsion by mechanical agitation, the increase in viscosity of the W/O emulsion results in a reduction of shear force for the oil phase. As discussed in a previous paper,[10] the size of the resulting liposomes was dependent on the revolution number, i.e., the shear force, during preparation of the W/O/W emulsion. Consequently, as seen in Figure 5, the mean diameter of the resulting

A. Diethyl ether
B. Dichloromethane
C. Chloroform
D. *n*-Hexane
E. Benzene

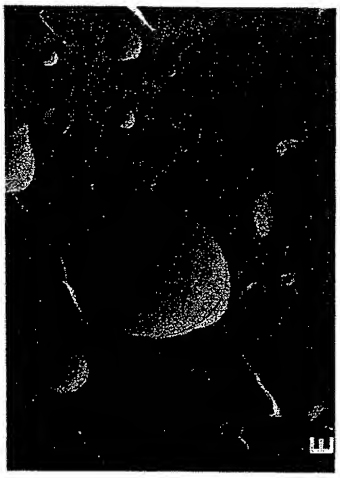

FIGURE 2. Freeze-fracture electron micrographs of MCV prepared with five different organic solvents. Bar indicates 200 nm.

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Figure 4 shows the relation between the mean diameter of liposomes determined for 900 particles on scanning electron micrographs and the boiling point of lipid solvents used for liposome preparation. As it can be seen, the mean diameter of liposomes decreased linearly as the boiling point of the solvent increased. In the MCV method, the W/O emulsion is poured into a dispersion medium (water), where it spontaneously forms a W/O/W emulsion, then the solvent of the oil phase is gradually lost by dissolution into the aqueous phase, finally producing liposomes. Thus, when removal of the solvent by evaporation occurs too quickly, the liposomes are produced without sufficient dispersion. Hence, the faster the removal of the solvent by evaporation, the larger the size of the resulting liposomes.

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FIGURE 2. Freeze-fracture electron micrographs of MCV prepared with five different organic solvents. Bar indicates 200 nm.

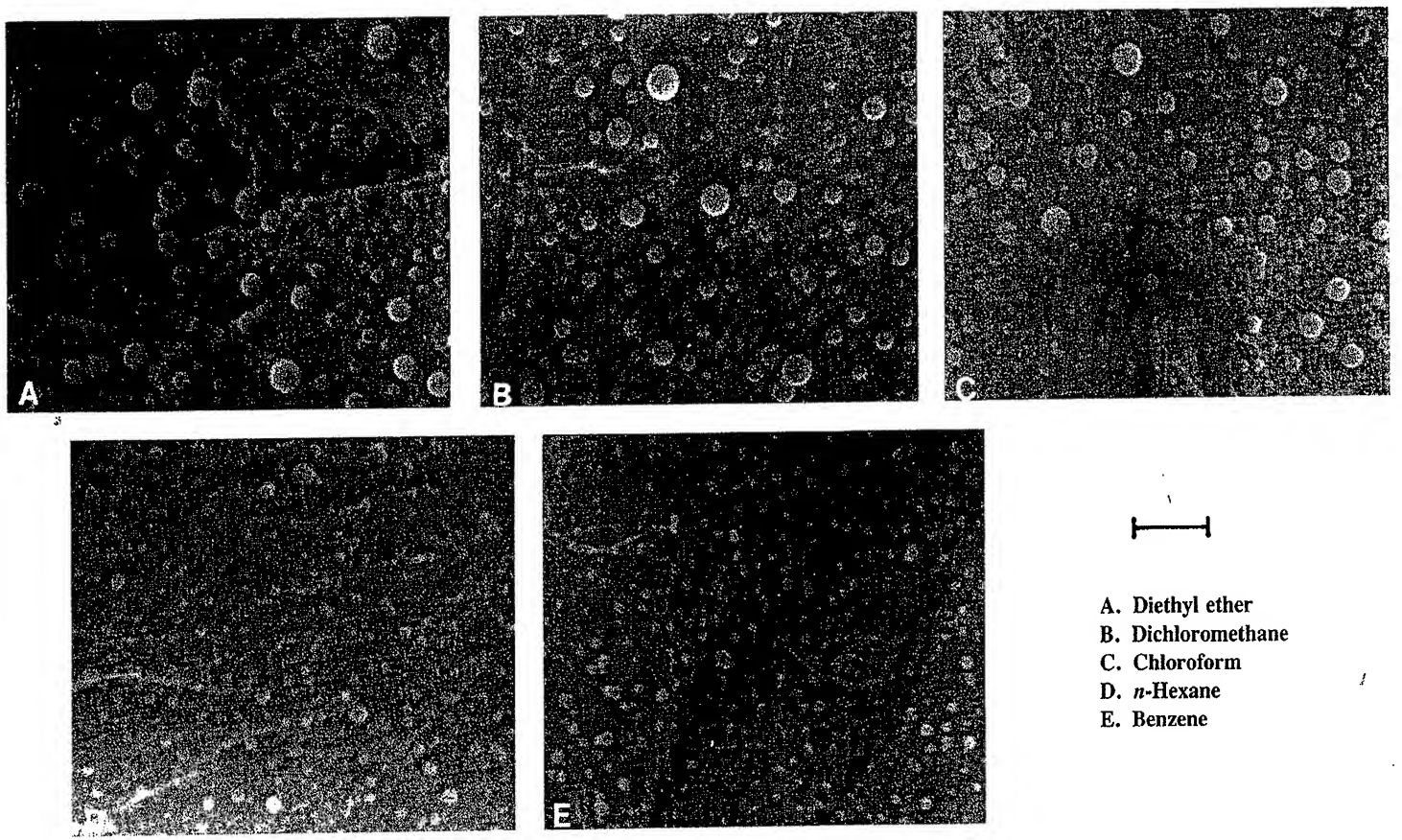

FIGURE 3. Scanning electron micrographs using the malachite green fixation technique for MCV prepared with five different organic solvents. Bar indicates 1000 nm.

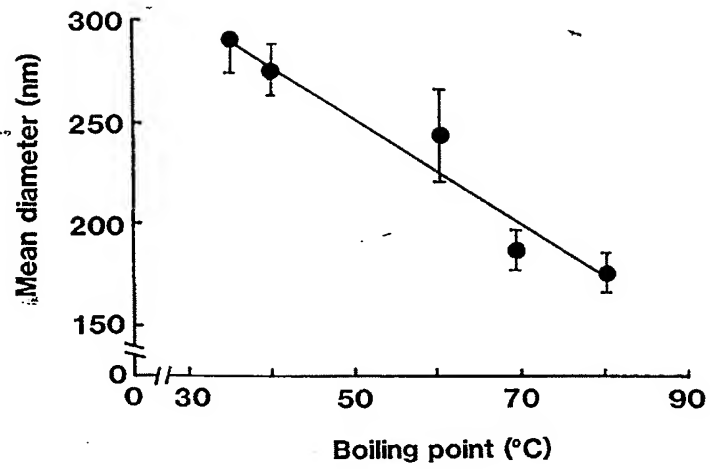

FIGURE 4. The effect of the boiling point of the lipid solvent on the mean diameter of resulting liposomes.

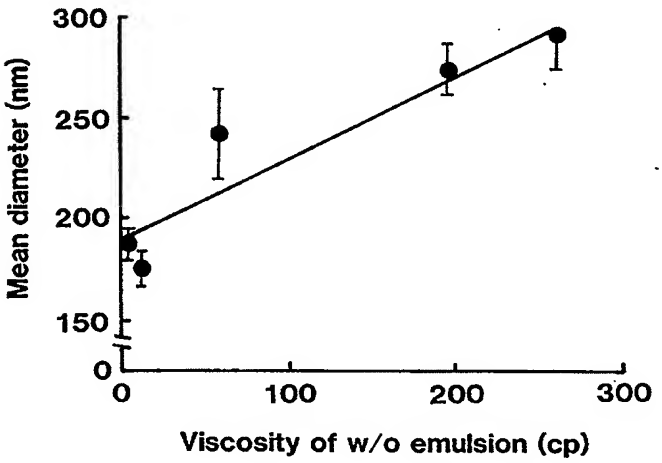

FIGURE 5. The effect of the viscosity of the W/O emulsion on the mean diameter of resulting liposomes.

liposomes was found to depend on the viscosity of the W/O emulsion. Moreover, it can be seen from both Figures 4 and 5 that the higher the boiling point of the lipid solvent used for preparing the W/O emulsion, the lower the viscosity of the latter became.

Some lipids, such as dialkyl phospholipids containing saturated fatty acids and hydrogenated phospholipid, are not completely soluble in most organic solvents, except for chloroform or mixed solvents containing an adequate amount of methanol. However, the present MCV method is applicable to a

FIGURE 3. Scanning electron micrographs using the malachite green fixation technique for MCV purposes. Bar = 1000 nm.

wide variety of lipid components with appropriate lipid solvents. The percentage encapsulation of the marker (calcein) in the total liposomes prepared by the MCV method was more than 60% (not shown), irrespective of the lipid solvent used.

In summary, the MCV method was found to be very useful for producing liposomes of controllable particle size as drug carriers or biological model membranes. One potential application[24] of this MCV method has already been reported. When tetanus toxoid-loaded liposomes were orally administered to cats, an antibody equivalent to that found after subcutaneous inoculation of absorbed tetanus toxoid was obtained. Moreover, liposomes prepared by this MCV method containing an anticancer drug and magnetite were infused into rabbits, and more than 80% of the liposomes were found to be retained at a target site in the ear vasculature at a magnetic field strength of 4000 G.[25] Studies to characterize the various physicochemical properties of MCV in more detail, and to apply MCV both *in vivo* and *in vitro* are currently in progress.

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